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The Interaction between Polysaccharides and other Macromolecules

5. THE SOLUBILITY OF PROTEINS IN THE PRESENCE OF DEXTRAN

By T. C. LAURENT

Department of Medical Chemistry, University of Uppsala, Sweden

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Experimental studies on aqueous mixtures of hyaluronic acid and proteins have indicated that a solute with a large effective volume, such as hyaluronic acid, exerts a profound influence on the relationship between other macromolecular solutes and the solvent (Ogston & Phelps, 1961; Laurent & Ogston, 1963). Ogston & Phelps have interpreted the observed results as a steric exclusion of foreign particles from the hyaluronic acid network, and have shown that this effect is more pronounced the larger the particle. If such a mechanism is operative it would mean that hyaluronic acid affects the activity of other substances and thereby should alter their solubility. To test this hypothesis, the solubilities of different proteins have been measured in the presence of a polysaccharide. Dextran was selected rather than hyaluronic acid to avoid any charge interaction between the macromolecules.

EXPERIMENTAL

Materials

Dextran. Preparations of dextran, a polyglucose, were obtained from AB Pharmacia, Uppsala, Sweden. The two commercially available preparations, dextran-150 and dextran-500, have limiting-viscosity numbers of 36 and 48 ml./g., and weight-average molecular weights of 153000 and 450000 respectively. Through the courtesy of Dr K. Granath, three other preparations with higher molecular

weights were obtained, namely dextran I, dextran II and dextran III. These had limiting-viscosity numbers of 94, 117 and 142 ml./g., and molecular weights of 6.9×10^6 , 13×10^6 and 25×10^6 – 50×10^6 respectively. The manufacturer kindly determined the degree of branching and found that approx. 94% of the glucosidic bonds in the various preparations were (1→6) linkages.

Ficoll. Ficoll, which is a synthetic polysucrose with a very compact structure, was also obtained from Dr Granath. It had a molecular weight of approx. 10^6 and a limiting-viscosity number of 14 ml./g.

D-Glucose and L-tyrosine. These were of analytical grade.

Proteins. The same preparations of human serum albumin, human γ -globulin, human fibrinogen and bovine α -crystallin described by Laurent, Björk, Pietruszkiewicz & Persson (1963) and Laurent & Persson (1963) were employed. Their molecular weights are 69000, 160000, 341000 (see, for example, Phelps & Putnam, 1960) and 830000 (I. Björk, unpublished work) respectively.

Methods

Determination of the solubility of proteins. Stock solutions of proteins (3×10^{-2} – 10×10^{-2} g./ml.) were made up in water, 0.1M-phosphate buffer, pH 7.6, or 0.1M-acetate buffer, pH 4.7.

Stock solutions (100 ml.) of the dextrans, ficoll and glucose (7×10^{-2} – 12×10^{-2} g./ml.) were prepared by dissolving these compounds in a small volume of water; with dextran, this was done at 100°. Then the ammonium sulphate and in most experiments either phosphate buffer, pH 7.6, or acetate buffer, pH 4.7, were added, and the

solutions were made up to 100 ml. at 20°. The salt was added in one of two ways so as to obtain: (a) a specific molarity (M) of ammonium sulphate and buffer, or (b) a specific molality (m) of ammonium sulphate and buffer. To accomplish the latter, the partial specific volumes of dextran (0.611; Granath, 1958), glucose (0.620; Roth & Scheel, 1923) and ficoll (0.61; estimated) were used to calculate the volume occupied by the sugars. The amount of salt to be added was then calculated on the basis of the remaining volume from a known relationship between the molality of the salt and its weight per volume. In some cases the desired salt concentrations were obtained by dialysis of dextran solutions against the specified salt solutions. The solutions obtained by both dialysis and admixture of constituents were found to have identical effects on the solubility of albumin.

The concentrations of dextran, ficoll and glucose were varied by mixing the stock solutions with the appropriate buffer-salt solutions.

The experiments were generally carried out by mixing 1 ml. of protein solution with 7 ml. of a salt or salt-sugar solution at 0°. A control solution having the same composition except for the protein was prepared for each sample. The solutions were kept for 24–48 hr. at 4° and then spun in a refrigerated Spinco model L preparative ultracentrifuge at 20000 rev./min. for 30 min. in a rotor precooled to 4° to remove the precipitate that formed. The supernatants were read against the control solutions in a Beckman DU spectrophotometer at 280 $m\mu$. The concentrations of the protein have been expressed as extinctions at 280 $m\mu$.

Determination of the solubility of tyrosine. A volume of 8 ml. of dextran-500 (0.7×10^{-2} g./ml.) dissolved in 0.1M-phosphate buffer, pH 7.6, was added to centrifuge tubes containing 10 mg. of tyrosine. For each tube a control was prepared containing no tyrosine. The tubes were shaken for 48 hr. at 4° and centrifuged as described above. The supernatant from each tube was diluted tenfold and its extinction was read against its control at 280 $m\mu$.

RESULTS

Selection of ionic strength for the experiments. To demonstrate the effect of polysaccharides on the solubilities of proteins, it was necessary to employ media in which their solubilities were decreased to a suitable level. Advantage was taken of the fact that the solubility of a protein decreases logarithmically with ionic strength (see, e.g., Cohn & Ferry, 1943). The solubilities of the proteins used in the present investigation were determined at various ammonium sulphate concentrations in the presence of 0.1M-buffer at pH 7.6 or 4.7 as shown in Fig. 1. From these results, salt concentrations that gave protein solubilities corresponding to extinctions of approx. 1.0 were selected for the experiments described below.

Comparison of the effect of dextran and glucose on the solubility of albumin. The solubilities of albumin in the presence of dextran-500 and glucose were compared to ascertain whether the effect of polysaccharide could be ascribed solely to its macro-

molecular character. The experiment was performed in 2.6M-ammonium sulphate and 0.1M-phosphate buffer, pH 7.6. There was a marked decrease in the solubility of albumin in the dextran solution but no change occurred in glucose (Fig. 2).

It can be argued that the observed changes stem from an exclusion of salt from the solvent within the domain of the dextran molecules, for in this event the salt concentration in the remainder of the solvent would be effectively increased and the protein solubility consequently diminished thereby. The remaining experiments have therefore been carried out in such a manner that the salt concentration in the solvent is constant, i.e. at constant molality. As shown below, when this is done, the solubility of albumin and other proteins in the presence of dextran does not decrease to the same extent. In experiments with glucose, an increased solubility of both albumin and γ -globulin was found with increasing sugar concentration.

Effect of dextran on the solubility of albumin at various ionic strengths. Fig. 3 shows the logarithm of the solubility of albumin in the presence and absence of 6×10^{-2} g. of dextran-500/ml. as a function of the ammonium sulphate concentration. No buffer was used in these experiments. The relative decrease in solubility seems to be independent of ionic strength.

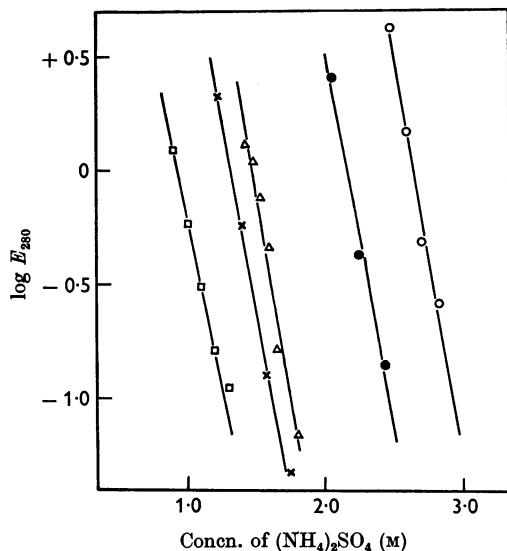


Fig. 1. Logarithm of the solubility of various proteins as a function of ammonium sulphate concentration. All solutions contain 0.1 M-buffer. The solubility is expressed as the extinction at 280 $m\mu$ of the saturated protein solutions. \square , Fibrinogen, pH 7.6; \times , γ -globulin, pH 7.6; \triangle , α -crystallin, pH 7.6; \circ , albumin, pH 7.6; \bullet , albumin, pH 4.7. Details are given in the text.

Effect of dextran on the solubility of albumin at various pH values. The relative decrease in the solubility of albumin in the presence of dextran-500 in 3.3 *m*-ammonium sulphate, and in 0.13 *m*-acetate buffer, pH 4.7, and 2.4 *m*-ammonium sulphate is about the same (Fig. 4).

Influence of the molecular weight of dextran on the solubility of albumin. The relative decrease in the

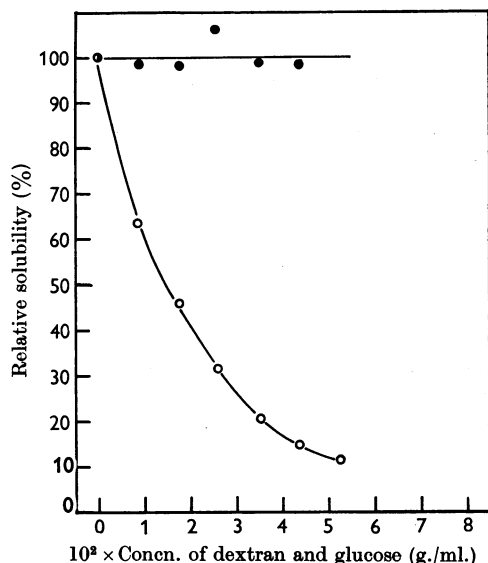


Fig. 2. Relative solubility of albumin in the presence of glucose (●) and dextran-500 (○). The ordinate expresses the solubility in the presence of the sugar relative to the solubility when no sugar is present. Details are given in the text.

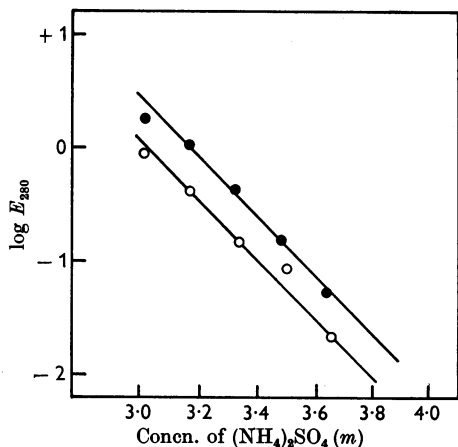


Fig. 3. Solubility of albumin in the presence (○) and absence (●) of 6×10^{-3} g. of dextran-500/ml. as a function of the ammonium sulphate concentration.

solubility of albumin in dextrans of various molecular weights is shown in Fig. 4. The concentration of ammonium sulphate used was 3.3 *m*. No buffer was present. Though the higher-molecular-weight dextrans may have a slightly larger effect, it is hardly significant. The scatter in the results is probably due to the difficulty in spinning down the protein precipitates through the highly viscous dextran solutions without creating a concentration gradient of dextran in the centrifuge tube.

Influence of ficoll on the solubility of albumin. These experiments were carried out at pH 4.7 in 2.4 *m*-ammonium sulphate because the polysaccharide is insoluble at higher salt concentrations. Ficoll had a slight solubilizing effect on albumin (Fig. 4).

Effect of dextran on the solubility of various proteins. The solubilities of proteins with different molecular weights were tested in the presence of dextran-500. The results are presented in Fig. 5. The ammonium sulphate concentration in the solvent was 3.15 *m* in the experiments with serum albumin, 1.4 *m* with γ -globulin, 0.95 *m* with fibrinogen and 1.65 *m* with α -crystallin. All determinations were made in the presence of phosphate buffer, pH 7.6, which in the four experiments was 0.14 *m*,

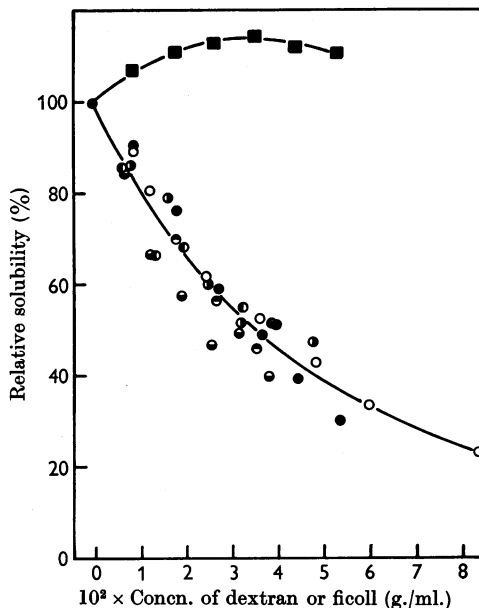


Fig. 4. Relative solubility of albumin in the presence of dextrans of various molecular weights and of ficoll. Five experiments were made in 3.3 *m*-ammonium sulphate [dextran-150 (○); dextran-500 (○); dextran I (●); dextran II (◐); dextran III (◑)], and two in 0.13 *m*-acetate buffer, pH 4.7, and 2.4 *m*-ammonium sulphate [dextran-500 (●); ficoll (■)].

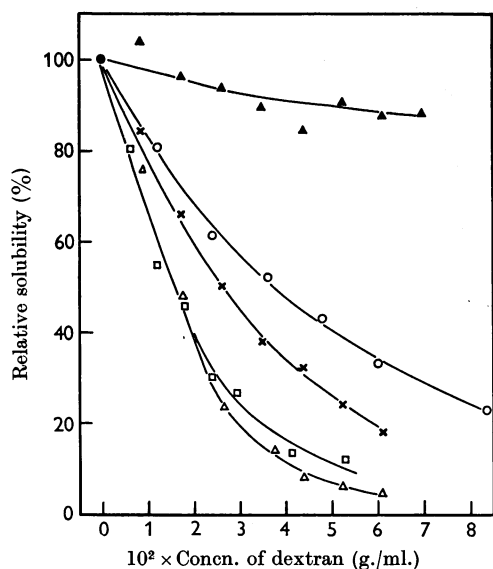


Fig. 5. Relative solubility in the presence of dextran-500 of tyrosine (▲), serum albumin (○), γ -globulin (×), fibrinogen (□) and α -crystallin (△).

0.12 *m*, 0.11 *m* and 0.12 *m* respectively. For comparison, the solubility of a low-molecular-weight compound, tyrosine, is also shown. It is obvious that the effect of dextran on the solubility of proteins increases with increasing size of the proteins.

DISCUSSION

The object of the present investigation was to evaluate the influence a polysaccharide might have on the solubility of other macromolecular substances. This is important in connective tissue, where the glycosaminoglycans are among the major constituents of these structures. If they have a role in regulating the deposition of insoluble material, polysaccharides may take part in reactions such as collagen-fibre formation, bone mineralization, precipitation of antigen-antibody complexes and deposition of lipid material.

To have as simple an experimental system as possible, an uncharged polysaccharide, dextran, has been used, as a first approach, instead of a highly charged natural glycosaminoglycan. The experiments were performed in media of high ionic strength so as to obtain a reasonably low protein solubility. This, however, raises the question whether changes in the solubility of a protein in the presence of dextran are due to the effect of dextran on the activity of the salt, for the solubility of proteins at high ionic strength is very sensitive to even small changes in salt activity (Cohn & Ferry, 1943;

and Fig. 1). To eliminate this contingency, all experiments (except those shown in Fig. 2) were carried out at constant-molal salt concentrations.

Fig. 2 shows the solubility of albumin in the presence of dextran and glucose. The marked effect of dextran, a polyglucose, must be ascribed to its macromolecular properties, as the monomer itself is without effect. The degree of polymerization of the macromolecule, once it exceeds a certain minimum, has little significance (Fig. 4). This agrees with the assumption that the phenomenon is a result of an exclusion of protein from the volume occupied in solution by dextran; for the excluded volume should depend on total chain length per unit volume rather than the size of individual dextran molecules. A very compact molecule, e.g. ficoll, should have a small excluded volume and the results are in agreement with this expectation.

Additional evidence supporting the steric exclusion hypothesis is found in the experiments that show that the relative decrease in solubility of albumin in dextran is independent of its absolute solubility (Fig. 3), the salt concentration (Figs. 3 and 4) and the pH (Fig. 4). The excluded volume should be a function solely of the dextran concentration and independent of the albumin concentration or any other factor.

The strong dependence of the solubility of a protein on its size shown in Fig. 5 further substantiates the original hypothesis. In general, the larger the protein, the less soluble it is in the dextran. The fact that the solubility of fibrinogen (mol.wt. 340 000) is diminished almost to the same extent as the solubility of the much larger α -crystallin (mol.wt. 830 000) may be due to the high degree of asymmetry of the fibrinogen molecule, which increases its effective size.

The limited solubility of even larger particles in polysaccharide solutions has been noted by Cohen (1942), who showed that plant viruses and haemocyanin could easily be crystallized in the presence of a large number of polysaccharides.

In a linear polysaccharide chain with an assumed effective radius of 4 Å and a density of 1.6, the volume excluded for albumin [effective radius 34 Å (Laurent *et al.* 1963)] should be about 55 ml./g. at low polysaccharide concentrations when calculated as described by Ogston (1958). From the present experimental results it is possible to calculate the excluded volume for albumin at various dextran concentrations. At 2×10^{-2} and 8×10^{-2} g. of dextran/ml., the excluded volume is 16 and 9.6 ml./g. respectively. The volume excluded by dextran at low concentration is thus only one-third of the value calculated for a linear chain. This is probably due to the extensive branching (one branch point on every fifteenth glucose unit) that gives dextran a relatively compact structure. The

excluded volume of a linear polysaccharide is therefore expected to be larger. That the excluded volume decreases with increased dextran concentration is expected because of the increased overlapping of the neighbouring molecules.

There may be other explanations for the effect of dextran on the solubility of proteins. Formation of a complex between the protein and the polysaccharide, which had a lower solubility than its constituents, could account for some of the results that have been observed. A charge interaction is unlikely because the dextran is essentially uncharged, and, further, the effect persists even at very high concentrations of electrolytes and is independent of the electrolyte concentration and pH over a wide range. Other types of chemical interaction are equally improbable, for the effect seems to vary only with the dextran concentration while being independent of the albumin concentration even at very low concentrations.

In addition to the importance of this phenomenon in biological systems, it may have practical application in the fractionation of high-molecular-weight compounds.

SUMMARY

1. In media of high ionic strength the presence of dextran significantly decreases the solubility of albumin, γ -globulin, fibrinogen and α -crystallin.
2. The relative decrease in solubility is a function of the size of the protein and the concentration of dextran.
3. The effect on albumin is independent of the degree of polymerization of dextran (mol.wt. greater than 150000), the pH, the absolute salt concentration or the absolute protein concentration.

4. The results are discussed in terms of a large effective volume of dextran.

5. The importance of the phenomenon for natural polysaccharide-protein systems is emphasized.

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The Preparation of Crystalline Forms of Ferricytochrome b_2 and Ferrocyclochrome b_2

BY R. K. MORTON AND KATHRYN SHEPLEY

Department of Agricultural Chemistry, Waite Agricultural Research Institute, University of Adelaide, South Australia

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Cytochrome b_2 is the L-lactate-cytochrome c oxidoreductase (EC 1.1.2.3) of baker's yeast, first partly purified and described by Bach, Dixon & Zervas (1946). A method for purification and crystallization was developed by Appleby & Morton

(1954), who showed that the crystalline enzyme was a flavohaemoprotein containing a DNA component (Appleby & Morton, 1954, 1959a, b, 1960; Appleby, Morton & Simmonds, 1960). Non-crystalline preparations of the enzyme, apparently